LIPID NERVE SHEATHS IN INSECTS AND THEIR PROBABLE RELATION TO INSECTICIDE ACTION

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INTRODUCTION

This study is an outgrowth of previous work in which it was shown that toxic petroleum oils cause a degeneration that seems largely a separation of the individual nerve cells and their processes (Richards, 1941a). While studying the action of toxic oils it was noted that oil solvents marked with Sudan dyes penetrated electively into the nervous system from tracheæ. Correlating these two sets of data resulted in the hypothesis that, despite current beliefs to the contrary, insect nerve cells and their processes are surrounded and insulated by some lipid material. The present paper confirms this idea. It shows that insect nerves are surrounded by bound lipid sheaths, and further that the penetration of oil solvents, and so presumably of oils, is correlated with the distribution of this lipid, and that the destruction of the lipid nerve sheath is at least one of the effects of certain neurotoxic insecticides.

OBSERVATIONS ON THE NERVE SHEATHS

When mosquito larvæ (*Culex pipiens;* Richards, 1941b) come into contact with a layer of xylol the fluid readily enters the spiracles and more or less fills the tracheal system. Such larvæ become paralyzed within a minute or two, and the heart stops beating in about 15 minutes. If the xylol is marked with Sudan dyes, it is found that in those segments in which the tracheæ are filled with xylol the nerve cord soon becomes intensely colored and stands out in contrast to the other tissues which remain uncolored. Obviously the marked xylol can penetrate from tracheæ into the nervous system more readily and in vastly larger quantity than it can into other tissues.

Dissection of such specimens shows that the color is more con-

centrated in fiber tract regions (Fig. 2). Only a relatively small amount can be discerned in the cell-body regions. Dissection of specimens in which the marked xylol is just beginning to penetrate into the nervous system shows that it diffuses rapidly from the small tracheæ into and then along the fiber tract regions (Fig. 3). This suggests the intermediation of some material in which considerable quantities of xylol are readily soluble, *i.e.*, a lipid.

Brains and nerve cords of larvæ dissected in 10 per cent formalin (4 per cent formaldehyde) and left in the formol for 24 hours can be readily stained with Black Sudan B[1] (Figs. 4–5). Such larvæ show only a diffuse stain in the fiber tract regions such as has been recently reported by Wigglesworth (1942) for mosquito larvæ fixed in Bouin's fluid. Short fixation in 10 per cent formalin is inadequate in the sense that subsequent staining in the alcoholic dye results in a partial release of lipid.[2]

The larval nervous system may be similarly stained by brief immersion of living ganglia in the alcoholic stain, but alcohol soon releases the lipid which then stains as dark particles. This experiment is most easily performed by carefully removing the top of the larva's head (in saline) and placing the part containing the intact nervous system in the alcoholic stain for about five minutes. After removal and rinsing, the dissection is completed in saline. The stain will not have penetrated completely to the subœsophageal ganglion, and a full series of effects will be obtained in a single brain (Figs. 6–7). In the exposed upper part the lipid will have been released by the alcohol and stained as particles, in the subœsophageal ganglion the lipid will be undetectable (and presumably unaffected), and in the intermediate

[1] For source and techniques see Hartman (1940). Staining of entire nerve cords takes only 5–15 minutes in the stain in 70% alcohol but several hours in a saturated solution of the stain in 50% alcohol. As mounting medium I have used Apathy's Gum Syrup. This is made by dissolving 50 grams of gum arabic and 50 grams of sucrose in 50 cc. of warm distilled water, filtering, cooling and then adding 1 cc. of formalin. For the fats of the adipose tissue this makes a good permanent mounting medium that does not require sealing (I have slides over three years old) but the nerve sheath lipids are soon altered in this as in other media tried.

[2] This might conceivably be due either to inadequate fixation or to the slow removal of one lipid fraction in 10% formalin. See Weil (1933).

parts a region will be found with diffuse staining which grades into particulate staining above and unstained tissue below.

Material fixed in 50 per cent alcohol and then stained with Black Sudan B shows all or almost all of the color located in particles (Fig. 8). These particles are concentrated in the fiber tract regions and are clearly *outside* the nerve fibers since by crushing the fresh whole mount they can be made to float around freely independently of the uncrushed cells. Smaller and less numerous particles also occur between the cells, giving indication of a lipid layer extending around the cells as well as around the processes.

The lipid particles released by alcohol are usually small, have irregular shapes (Fig. 10), stain intensely with Sudan dyes, and have a high melting point ($> 100°$ C.). Oblong particles exhibit negative birefringence with respect to their long axes. The material composing these particles is rapidly removed by high concentrations of ethyl alcohol (70 per cent and higher) and slowly by low concentrations of alcohol and even by water. The nerve sheaths also appear to be heat labile, for while osmium tetroxide heavily blackens the interfibrillar material in larvæ killed by heat (45° C. for 3 minutes), it does not do so in control animals (Figs. 11–13).

The lipid composing the nerve sheaths is clearly different from the lipids of the adipose tissue. The latter are not visibly affected by aqueous fixing fluids or by a temperature of 45° C., and, while they are soluble in 95 per cent alcohol, they are insoluble in 70 per cent alcohol.

To determine whether or not the lipid nerve sheaths occur generally throughout the group a miscellaneous set of insects were collected, opened and fixed in 10 per cent formalin, and then the dissected nervous systems stained with Black Sudan B and examined as partially crushed whole mounts. Species examined were: *Gryllus assimilis* (Orthoptera), *Phymata erosa* (Hemiptera), *Harpalus* sp. (Coleoptera), *Bombus* sp. (Hymenoptera) and *Eristalis tenax* (Diptera). With all of these species the same results were obtained as have been recorded above for formalin-fixed mosquito larvæ. It would seem from these observations that lipid nerve sheaths are of general occurrence among insects.

The preceding data also suggest that the lipids of the nerve sheath are, at least in part, bound phospholipids (phosphatids). To check this, extracts were made from brains (supraœsophageal ganglia) and subœsophageal ganglia of honey bees (workers). This work is purely qualitative and based solely on solubilities; as such it is to be considered as preliminary although indicative. Brains (usually including the subœsophageal ganglia) were removed from normal bees and carefully cleaned of surrounding tissues. The adhering tracheal sheath was peeled off to insure removal of all pieces of the head glands, the œsophagus removed as a unit, and the optic and ocellar nerves severed to prevent inclusion of any eye pigments. Dissections were performed in lots of five or six, and the nervous tissue then dried in a vacuum where it was gradually accumulated (in darkness). From 225 such dissections, 53 milligrams (dry weight) of pure nervous system were obtained. This was extracted with dry ethyl ether (in darkness), filtered, an excess of dry acetone added to the filtrate, and the tube placed in a refrigerator overnight. A fine white precipitate resulted. This was filtered and the residue after drying redissolved in ether and concentrated on a warm water-bath. Portions of the redissolved material were dried on clean slides and tested for alcohol solubility: a majority of the material dissolved in 95 per cent ethyl alcohol but a small amount was insoluble. The ether-acetone filtrate was likewise concentrated and portions dried on clean slides; a considerable residue was present after drying. This latter residue is readily soluble in 95 per cent ethyl alcohol and slowly soluble in 70 per cent alcohol. The original ether extract then shows acetone-soluble and acetone-insoluble fractions, both of which can be stained with Black Sudan B in 50 per cent alcohol. The acetone-soluble fraction is also soluble in ethyl alcohol. The acetone-insoluble fraction is divisible into an alcohol-soluble and a smaller alcohol-insoluble fraction. Using the data given by Page (1937), the above suggests the presence of considerable amounts of cholesterol (acetone- and alcohol-soluble) and lecithin (acetone-insoluble, alcohol-soluble) and small amounts of cephalin (acetone- and alcohol-insoluble). While some of these substances were doubtless extracted from the nerve cells themselves, comparison with

the histochemical data indicates clearly that the nerve sheaths contain the same lipids as the extracts.[3]

RELATION OF THE NERVE SHEATHS TO INSECTICIDE PENETRATION

The elective penetration of stained xylol into the nervous system (Fig. 1) not only indicates the presence of lipids but also shows that these lipids are of prime importance in the penetration of oil solvents. Specimens dissected during the penetration process show the course of diffusion of the stain from the tracheæ into and then along the fiber tracts (Fig. 3). Presumably this relationship will also be true for toxic oils and oil-borne toxins.

RELATION BETWEEN THE PENETRATION OF XYLOL AND PARALYSIS

Specimens in which by chance the penetration into successive ganglia is progressive and not too rapid show paralysis of those segments in which the ganglia are stained while the more anterior segments (farther from the spiracles) are still reacting. There is then a direct correlation between the penetration of xylol into a ganglion and the prompt paralysis of that segment. Such a correlation is commonly pointed out in insecticide studies but in this case penetration of the toxin can be observed directly instead of assumed.

Unfortunately, the destructive action of alcohol makes it difficult to study the action of insecticides on the nerve sheaths. Techniques are now being studied in a search for better procedures.

Xylol is obviously a nerve poison. Its rapid penetration into the nervous system is correlated with and so presumably conditioned by the lipid nerve sheaths. But it does not seem to destroy these sheaths, at least not to the extent that toxic petroleum oils and postmortem degeneration do (Richards, 1941a). Xylol must also penetrate (more slowly and in smaller quantity) into other tissues as is shown by the cessation of heartbeat in approximately 15 minutes. Quite likely the toxic effect of xylol is a general one

[3] Comparison with the data given for crustacean nerve sheaths by Schmitt, Bear and Clark (1935) would suggest that cholesterol is also involved in the insect nerve sheath complex but the histochemical data given in the present paper are not sufficient to make any statement at this point.

and xylol seems to be a nerve poison largely because of the speed of its penetration into the nervous system. The nature of the action of xylol on living cells is not known.

DISCUSSION

The definite demonstration of bound lipid nerve sheaths is new for insects (Richards, 1942). It has generally been considered that insect nerves lack myelin sheaths or any counterpart thereof (*e.g.*, Lindsay and Craig, 1942). Hanström (1928) does not even discuss the possibility of interneuronal material other than connective tissue. The same is true for the brief reviews by Clayton (1932) and Hilton (1942), and the papers on non-nervous elements by Scharrer (1939, 1941). When the present study was nearing completion Wigglesworth (1942) published a paper on insect nutrition in which he noted a diffuse staining with Black Sudan B in the central fibrous region of the nerve ganglia of mosquito larvæ but he did not carry the analysis further.

In invertebrates other than insects there is a growing literature on myelin-like or bound lipid nerve sheaths. The data are largely derived from optical studies on large peripheral nerves, and concern nerve fibers of Annelida (Young, 1937), Crustacea (Retzius, 1890; Schmitt, Bear and Clark, 1935; Bear and Schmitt, 1937; Chinn and Schmitt, 1937) and the Squid (Bear, Schmitt and Young, 1937).[4] Data presented herein on insects are largely (not exclusively) derived from the central nervous system, and are based on histochemical methods including extractions. However, the histochemical and optical data are probably comparable. At least in the mosquito larva bound lipid nerve sheaths of submicroscopic thickness are clearly indicated for both the central nervous system and for the individual fibers of the peripheral nerves. Preliminary observations on representatives of other orders of insects suggest, as would be expected, that bound lipid nerve sheaths will be found throughout the class Insecta.

The chemical identity of the lipid or lipids in the nerve sheath is still uncertain. The histochemical data and melting point sug-

[4] It might also be mentioned that even the so-called non-myelinated nerve fibers of vertebrates have similar lipo-protein sheaths (Schmitt and Bear, 1937). For a review of the literature on nerve sheaths see Schmitt and Bear (1939), and for more recent papers see Taylor (1940) and Holmes (1942).

gest a phospholipid. Ether extracts of bee brains can be separated into fractions, the solubilities of which suggest cholesterol, lecithin and perhaps small amounts of cephalin. Comparison of these data with the data of Schmitt, *et al.*, clearly suggests that the submicroscopic insect nerve sheaths are composed of one or more phospholipids (lecithin ?), and perhaps cholesterol, bound with protein.

The presence of bound lipid nerve sheaths is of histological value but their probable relation to the penetration of neurotoxic insecticides is of major interest in any study of the mode of action of these toxins. Insects differ fundamentally from vertebrates in that toxins cannot only reach the nervous system from the blood but also directly from the tracheæ. The entrance of oil solvents such as xylol into the nerve cord from the tracheæ is correlated with the distribution of this lipid and so presumably conditioned by it. It seems reasonable to assume that the same will be true for any oil or oil solvent that can penetrate tracheal walls.[5] In fact, any toxin, entering either from the tracheæ or blood, must traverse this bound lipid sheath to enter the nerve cells or processes. Partition coefficients would favor lipid-soluble materials.

The histopathological effects produced by toxic petroleum oils (Richards, 1941a) are consistent with the view that the destruction of the lipid nerve sheaths is intimately involved in the neurotoxic action of the oils. It seems likely that the same is true for the action of pyrethrins (see especially Klinger, 1936). Sheath degeneration also occurs as a relatively early postmortem change in asphyxiated mosquito larvæ (Richards, 1941a). But whether or not the sheath destruction is directly concerned in the production of paralysis and death is unknown. (See below.)

Another interesting analytical trend comes from the compari-

[5] In studying the effect of "Flit" on bees, Nelson (1927) reports the penetration of the stained mixture from tracheæ into the nerve ganglia, muscles and some of the malpighian tubes. The data are not strictly comparable to those presented in the present paper. However, the coloring of the ganglia is suggestive. And, in view of the long time factor (15 hours), the coloring of some other tissues is not surprising. Xylol must affect other tissues too since it causes a cessation of the heartbeat within 15 minutes, but only in the nervous system does it accumulate in sufficient quantity to visibly color the tissue by the time of death.

son of a series of papers which unfortunately deal with different species of insects and so must be correlated with caution. (1) Feldberg (1940) demonstrated phosphatase activity for bee venom by using it to prepare lysolecithin from purified lecithin. Bee venom appears to have the same action as cobra venom in producing lysolecithin in vertebrate tissues and in releasing histamine (see also Feldberg and Kellaway, 1937). Whether the toxic effect is produced by lysolecithin directly or through the intermediation of histamine is another question but need not be considered here. (2) It is well known that many parasitic or parasitoid wasps cause a true paralysis by stinging the ganglia of their arthropod prey. Hartzell (1935) has reported that the venom of the wasp, *Sphecius speciosus,* causes nerve lesions in the ganglia of the cicada, *Tibicen pruinosa.* (3) The present paper reports the presence of bound lipid nerve sheaths in insects, and gives data suggesting that the lipids are phospholipids including considerable amounts of some lecithin. (4) And, finally, the present paper points out that at least some of the types of insect nerve lesions reported by various authors seem interpretable as due to the breakdown of these phospholipid sheaths.

If these apparent correlations could all be demonstrated in one study, it would follow that paralyzing venoms probably act on arthropods by disrupting the lipid nerve sheaths and producing lysolecithin which in turn acts on the nerve cells (perhaps also indirectly). If this is true, another interesting point may be that the important destructive effects are not really the histologically visible lesions, because Feldberg (1940) has noted that the destructive effects of lysolecithin are not histologically demonstrable in the vertebrate adrenal gland.

The relationship of the data in the preceding two paragraphs to the action of neurotoxic insecticides is unknown. There is as yet no evidence that phosphatase activity is involved in insecticide action. Also neurotoxic insecticides kill whereas the venoms injected by wasps only paralyze.[6] Data from surgical operations show that mere elimination of parts of the central nervous system does not necessarily cause death (Kopeć, 1923; Metalnikov and Korvine-Kroukovsky, 1927). Data from the effects of toxic pe-

[6] Dosage rather than intrinsically different effect may be involved.

troleum oils suggest that destruction of the lipid nerve sheaths is an important act of these insecticides (Richards, 1941a). At least some of the data from the effects of pyrethrins likewise suggest destruction of the nerve sheaths (especially Klinger, 1936), but Wigglesworth (1941) reports that pyrethrins dissolved in liquid paraffin cause a far more general cellular destruction within ten days after paralysis,[7] and Hartzell and Scudder (1942) point out that the effects of pyrethrum are not necessarily confined to the nervous system. It seems to me certain that the lipid nerve sheaths are important factors in the penetration of neurotoxins and that these sheaths are commonly broken down by insecticides, but this does not mean that degeneration of the sheaths *per se* causes death or that other tissues cannot be penetrated and affected by neurotoxic insecticides.

SUMMARY

1. Insect nerve cells and their processes are surrounded and insulated by bound lipid sheaths of submicroscopic thickness. Solubility data suggest that the lipids are phospholipids, perhaps with the addition of cholesterol.

2. The lipid nerve sheaths are correlated with and so presumably condition the penetration of oil solvents into the nervous system from tracheæ. Presumably this finding will apply also to the penetration of toxic oils and oil-borne toxins.

3. One of the effects of certain neurotoxic insecticides seems to be the destruction of these bound lipid sheaths.

4. Some aspects of the relationships of these data to the study of insecticide physiology are discussed.

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[7] How much of this tissue dissolution precedes or accompanies the death of the cells is unknown. The mere fact that the heart is still beating does not necessarily mean that the nervous system is alive. The cells of the central nervous system may have been dead and undergoing a kind of postmortem degeneration for days. If this is true, the degree of degeneration of the nervous tissue is not necessarily any index of the type or extent of the insecticide action. The same criticism may be made of all other studies in which histopathology is used without a check on the physiological condition of the cells (see Richards, 1941a, pp. 182–183).

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PLATE III

Figure 1. Photomicrograph of two abdominal segments of mosquito larva treated with xylol stained with Black Sudan B. The body was cut longitudinally along one side, spread open, the gut removed, and mounted as a whole mount. Shows the intensely colored ganglia and their connectives. Also shows many small tracheæ containing stained xylol; these tracheæ branch around and through uncolored muscle, adipose and epithelial tissues. Magnification 75 ×.

Figure 2. Whole mount of brain (supraœsophageal ganglion) of mosquito larva treated with xylol stained with Black Sudan B. Note color is concentrated in the fiber-tract regions. Magnification 95 ×.

Figure 3. Whole mount of intact thoracic ganglia of a mosquito larva treated with xylol stained with Black Sudan B. Note metathoracic ganglion into which xylol is penetrating from the xylol-filled tracheæ on the left side. In the original mount the distinction between stain in the tracheæ and in the nerve tissue is much clearer. Magnification 270 ×.

Figure 4. Whole mount of removed thoracic and first abdominal ganglia of a mosquito larva fixed in 10% formalin for 37 hours and then stained with Black Sudan B in 70% alcohol. Magnification 120 ×.

Figure 5. Crushed whole mount of brain and subœsophageal ganglion of a mosquito larva fixed in 10% formalin for 37 hours and then stained with Black Sudan B in 70% alcohol. Air-filled tracheæ appear black, fluid-filled tracheæ are transparent. Black areas are fiber tract regions. Subœsophageal ganglion broken in half and twisted in mounting with result that one half lies above the brain, the other half below. Magnification 120 ×.

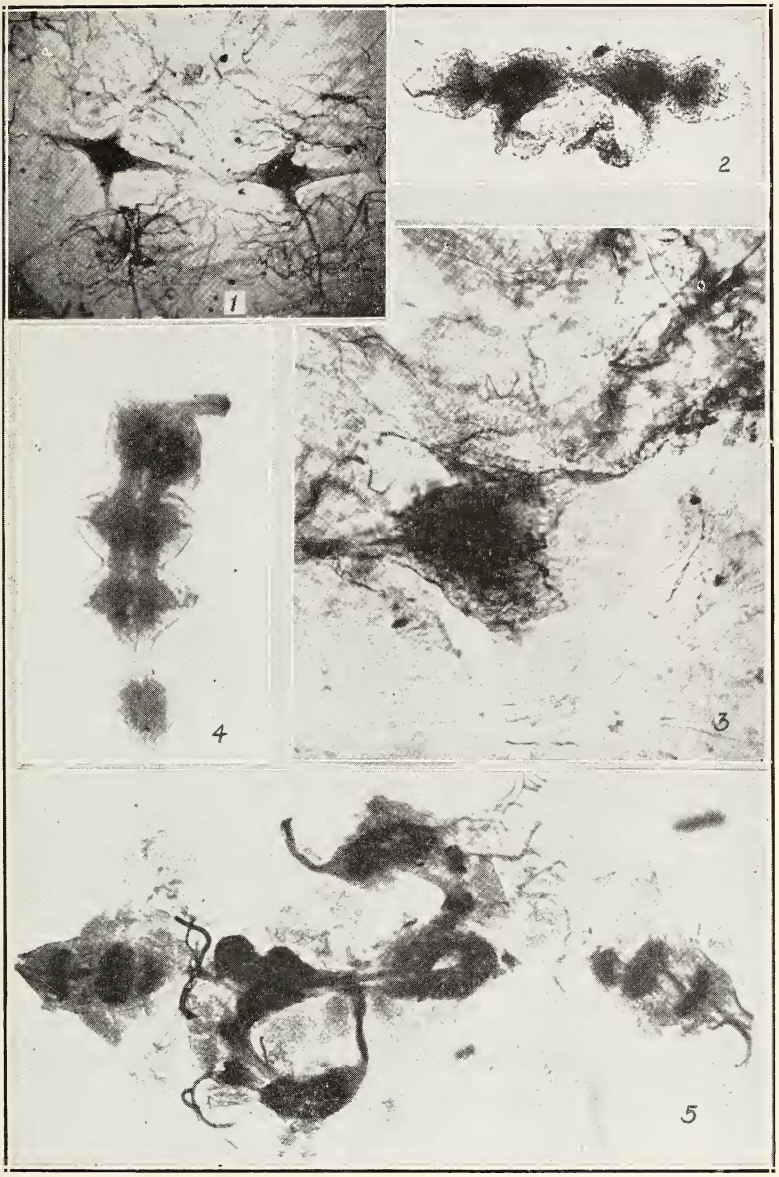
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PLATE IV

Figure 6. Portion of crushed whole mount of brain of mosquito larva showing diffuse stain along fiber tract leading to circumœsophageal commissures. Prepared by rapid staining of living brain in alcoholic stain. See text. Magnification 190 ×.

Figure 7. Another example of same of Fig. 6. Magnification 190 ×.

Figure 8. Portion of whole mount of brain fixed and stained in a saturated solution of Black Sudan B in 50% ethyl alcohol (6 hours). Stain mostly in free particles of various sizes. Note some particles in cell-body regions although bulk of stain is in fiber tract regions. Magnification 190 ×.

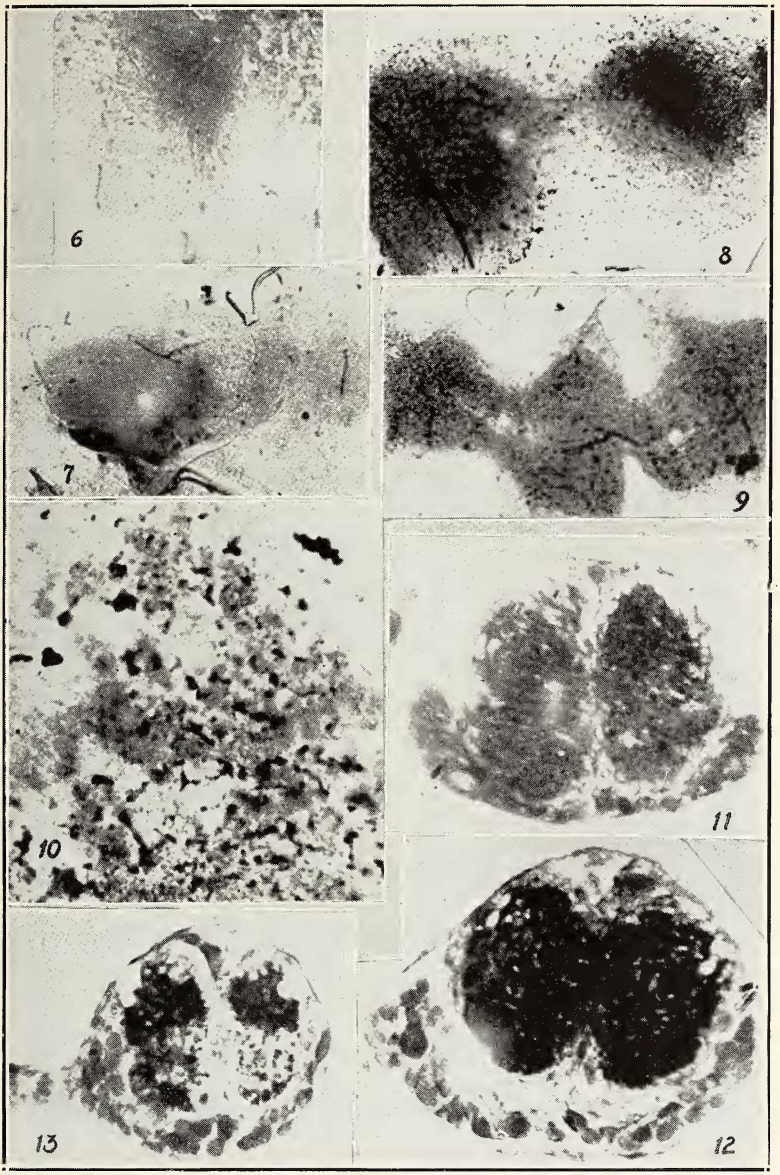
Figure 9. Whole mount of thoracic ganglia of larva fixed and stained in a saturated solution of Black Sudan B in 70% ethyl alcohol (5 minutes). Stain approximately half in free particles, half diffuse. Magnification 180 ×.

Figure 10. Higher magnification of released stained particles in a crushed whole mount of a brain fixed in 50% alcohol (15 hours) and then stained. Note that particles lying free in the mounting medium retain their irregular shapes. Magnification 250 ×.

Figure 11. Section of abdominal ganglion of normal larva fixed in 1% osmic acid for 24 hours. All parts of the nerve cord are colored a uniform light brown (same intensity as in the muscles). Magnification 340 ×.

Figure. 12. Section of abdominal ganglion of a larva killed by 3 minutes at 45° C. and then fixed in 1% osmic acid for 24 hours. Nerve cell bodies are colored a light brown (as in controls) but the fiber tracts are intensely blackened between the fibers. Magnification 340 ×.

Figure 13. Another section from a different specimen of same lot of larvæ as Fig 12. Note that the blackening of the fiber tracts is incomplete on one side in this particular section. Magnification 340 ×.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